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TITLE: The Blood-Testis Barrier and Male Sexual Dysfunction following Spinal Cord Injury

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14. ABSTRACT A majority of males exhibit a profound loss of fertility following spinal cord injury. While the mechanisms underlying this loss have been discussed for decades, recently my lab discovered that spinal trauma produces a significant loss in integrity of the blood-testis-barrier; a protective multi-cellular structure that maintains immune privilege of the highly-antigenic sperm and sperm cell-containing compartments within the testis. We also demonstrated that once failed, the BTB remains permeable, essentially for the life of the subject. The goal of our proposal has been two-fold: 1) to develop a greater understanding of the molecular, biochemical and structural pathologies underlying BTB breakdown post-SCI, and 2) to determine whether a novel therapeutic, recently identified in our laboratory, can help preserve BTB integrity when introduced during the acute phase of SCI using a clinically-relevant rat spinal contusion model. We have found that the drug, licofelone, preserves blood-spinal cord barrier integrity and enhances locomotor function in rats when given early following injury. During this second year, we have performed all planned spinal cord injuries (24 hour out to 90 day time points); collected testis tissues and have sent samples out for metabolomic analysis and gene array studies. We describe metabolomic, genomic and cellular pathologies occurring within the testes after SCI as well as data suggesting a beneficial role of licofelone in attenuating such changes.					
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Introduction:

Males who have received a spinal cord injury (SCI) face a lifetime of sensory and motor deficits. In addition to these well-described pathological outcomes, a majority of men will also experience a profound loss of fertility. This should be clearly understood to be separate from SCI-dependent erectile dysfunction that is due to a loss of neural input into the male sexual organs. SCI-dependent male infertility is characterized by a significant reduction in numbers and quality of functional sperm. The mechanism(s) underlying this deficit has previously been unknown. My laboratory has explored the effects of spinal trauma on tissues that exhibit “barrier” properties, or properties in which specialized tissues regulate the flow of materials from the blood stream into compartments throughout the body that are “immune privileged”. Our focus has been on the blood-spinal cord-barrier (BSCB) and how trauma collapses this important spinal vascular specialization; producing an environment that encourages long-term inflammatory conditions. We recently asked whether spinal trauma had any effect on the blood-testis-barrier (BTB), a specialized set of cellular structures located within the testis which protects sperm (immature through mature) as well as sperm precursor/stem cells from the immune system. We reported that a contusive injury to the rat spinal cord causes a profound and sustained loss of BTB integrity; resulting in the formation of both inflammatory and pro-oxidative conditions within the sperm-producing compartments. In addition, we detected the loss of structural elements that comprise the BTB as well as significant cell death and immune cell infiltration. The goal of this project is to: 1) further elaborate the early and long-term biochemical, molecular and structural deficits to the BTB elicited by spinal trauma, and 2) determine whether these pathological changes can be prevented or at least minimized by pharmacological modulation. We have recently found that a novel anti-inflammatory drug, licofelone, provided significant protection to the BSCB when administered to rats orally during the acute phase of SCI. Licofelone is a first generation anti-inflammatory drug that targets BOTH cyclooxygenase AND 5-lipoxygenase pathways of arachidonic acid metabolism; the two main pathways used to generate arachidonic acid-derived pro-inflammatory compounds (prostaglandins and leukotrienes, respectively). In the second aim of the current project (and a primary target of this year’s annual report), we describe how acute licofelone treatment results in attenuation of SCI-dependent pathological events that influence inflammation and oxidative stress. More importantly, we report that the attenuation of these pathological events resulting from early, acute licofelone treatment produce a sustained beneficial effect on these SCI-induced pathologies within the testes long after cessation of licofelone treatment. We had originally only requested two years of funding for this project. We are now asking for a No Cost Extension (NCE) to utilize remaining funds to perform subsequent validation of metabolomic and genomic changes as well as assess the effects of licofelone treatment on both acute and chronic sperm-associated antigens. Our results to date, however, are intriguing and suggest that SCI produces a wide range of pathologies, both acute as well as sustained over a long time period, within the testes. Our goal is to determine whether licofelone treatment can be subsequently translated as a novel therapeutic for the treatment of male infertility following SCI.

Keywords: Blood-testis-barrier (BTB), spinal cord injury (SCI), blood-spinal cord-barrier (BSCB), testes, testis, inflammation, oxidative stress, metabolism, genomic, metabolomic

Accomplishments:

What were the major goals of the project? The overall hypothesis and aims of this proposal were/are as follows:

Hypothesis: SCI induces biochemical, structural and functional changes within the BTB that contribute to a loss of sexual function in males. We further hypothesize that the application of a novel anti-inflammatory intervention will protect both BTB integrity and male reproductive capabilities.

Specific Aim 1: Explore the molecular, biochemical and structural changes that occur to the BTB as a function of time following the delivery of a clinically-relevant spinal contusion injury.

Specific Aim 2: Determine whether treatment with the new generation anti-inflammatory drug, licofelone, can protect BTB integrity and enhance germ cell and sperm viability over time following SCI.

We estimate that we are upwards of 80% complete with both aims. We are proposing to validate several of the observed metabolomic and gene array changes using commercially-available ELISA kits, Western blot and qPCR in our No Cost Extension Request (pending). In addition, we have added personnel, Lt. Ryan Fortune, who is an MD/PhD student in Dr. Grill's laboratory. Ryan has been working on this project through funding via a UT graduate student fellowship mechanism. He has performed the majority of the spinal cord injury studies in rats and has assisted Dr. Grill in all endstage tissue collections. He is currently performing both metabolomic, genomic and other endstage assessments/quantifications are part of his thesis work.

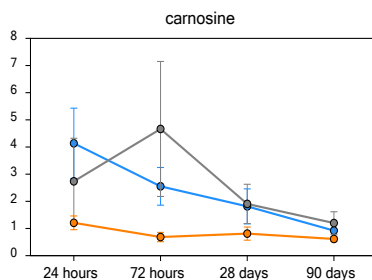
What was accomplished under these goals? (during the 2013-2014 period):

We will recount progress described in prior progress reports during the 2013-2014 period as well as describe new data collected prior to the end of the 2014 annual period.

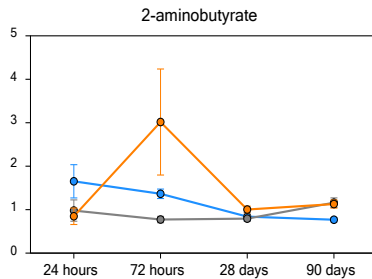
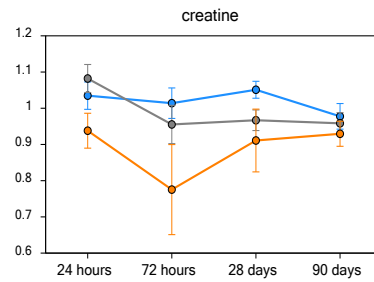
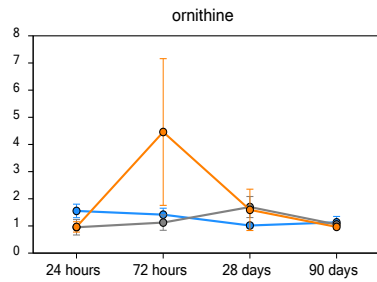
Aim 1:

Metabolomic studies: A single testis from each subject (SCI, SCI-sham or naïve-control) were collected at 24, 72 hours, 28 and 90 days from subjects that were euthanized, decapitated and tissues collected without transcardial perfusion. These tissues were snap-frozen before being sent to Metabolon, Inc. for Mass Spectrometry analysis.

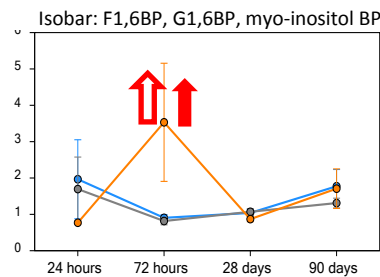
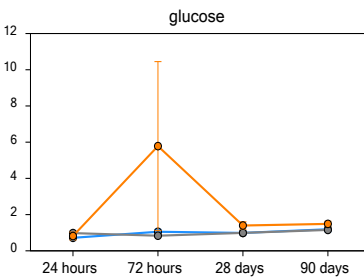
- We report a significant rise in prostaglandin E2, a pro-inflammatory mediator, in the testis of injured subjects at 24 hours. Interestingly, testis-concentrations of carnosine, an amino acid dipeptide with anti-oxidant properties, is suppressed significantly at 24 and 72 hours with suppression approaching significance at 28 and 90 days, suggesting a long-term pro-oxidative environment within the testis of rats following SCI.*



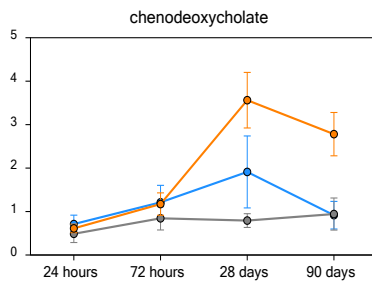
- We detect an increase in mediators such as valine, leucine, isoleucine, glutamine (etc), as well as other compounds that suggest a wave of cellular apoptosis that peaks at around 72 hours in the testis post-SCI.*
- Increased ornithine and 2-aminobutyrate and 2-hydroxybutyrate and decreased levels of creatine and creatinine suggests deficits in the urea metabolic cycle and energy metabolism in the testis at 72 hours post-SCI.*



- Aberrant glucose metabolism is also detected between 24 and 72 hours post injury



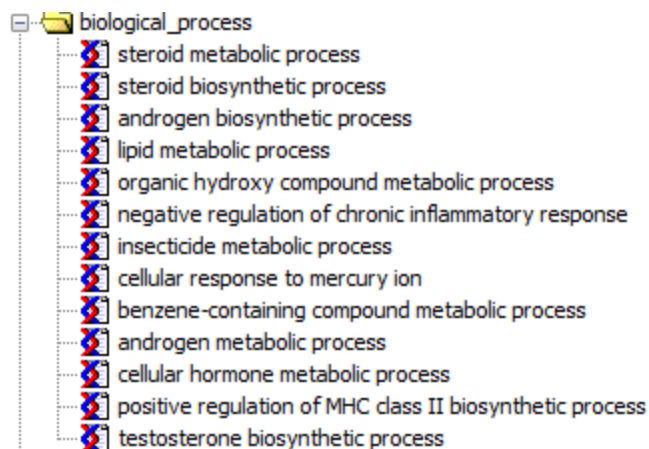
By 90 days, we observe altered metabolites of the bile acid pathway; specifically cholate and chenodeoxycholate which may suggest aberrant cholesterol metabolism.



Microarray studies:

One testis per subject was used for the metabolome study described above. The other testis from each subject was used for the gene microarray study. RNA preparation was performed in my laboratory. RNA samples were then provided, blind, to Dr. Loose who is Co-PI of this project as well as Director of the Molecular Core in the Department of Integrative Biology and Pharmacology. Samples were run on an Agilent Gene Array with subsequent cluster analyses performed using BRB Array tools and Pathways Analysis software. Significant differences in gene expression were considered with a minimum 2-fold change and significance at the $p < 0.05$ level.

- This represents our initial assessment of the gene array data. At 24 hours, we detect significant decreases in the following clusters of genes within the testis. You will note that many of these genes are associated with steroidogenesis and the regulation of inflammation.



We have also performed our initial analyses of gene changes at 90 days post-SCI and report significant increases in the following clusters:

GO ACCESSION	GO Term
GO:0050920	regulation of chemotaxis
GO ACCESSION	regulation of cell-substrate adhesion
GO:2000508	regulation of dendritic cell chemotaxis
GO:2000510	positive regulation of dendritic cell chemotaxis
GO:0050795	regulation of behavior
GO:0002690	positive regulation of leukocyte chemotaxis
GO:0005576	extracellular region
GO:0002688	regulation of leukocyte chemotaxis
GO:0010811	positive regulation of cell-substrate adhesion
GO:0002687	positive regulation of leukocyte migration
GO:0050921	positive regulation of chemotaxis
GO:0030155	regulation of cell adhesion
GO:0048520	positive regulation of behavior
GO:0002685	regulation of leukocyte migration
GO:0090023	positive regulation of neutrophil chemotaxis
GO:0071624	positive regulation of granulocyte chemotaxis
GO:0090022	regulation of neutrophil chemotaxis
GO:0045785	positive regulation of cell adhesion

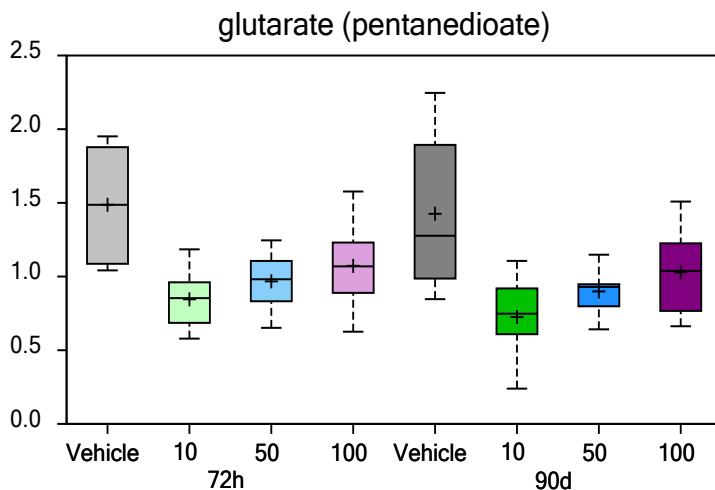
You will note that many of these clusters are tied to the cellular immune response and suggest an ongoing elevation in cross-talk between the testis and the immune system. This may support our hypothesis that a reduction in BTB integrity by SCI may result in a long-term autoimmune response against one's sperm producing testicular compartments.

Aim 2: We are still awaiting the genomic data generated from Aim 2; specifically to determine whether licofelone treatment abrogates any of the gene changes in the testes suggestive of a pathological response to spinal trauma. However, here we restate previously presented metabolomic data that suggests licofelone-mediated benefits to SCI-induced pathological responses to metabolism in the testes:

This funding period revolved around our analysis of the metabolomic data generated by Metabolon, Inc. Metabolon had informed us that the array of targets used to assess our Aim 2 specimens was larger, containing greater numbers of biochemical pathways compared to the metabolomic assessment performed previously on testes samples provided from Aim 1. As a reminder, adult, male Sprague-Dawley rats received a moderate spinal contusion injury. One cohort received only vehicle as oral treatment whereas other cohorts received oral licofelone at 10, 50 or 100 mg/kg, once a day, for 14 days starting 3 hours post injury. Subjects were euthanized at 72 hours and 90 days, testes removed and one testis per subject sent to Metabolon for Mass Spec analysis. Metabolon made available their results to us which we are/and continue to go through to identify both SCI-dependent pathological events occurring within the testis as well as attenuation of such

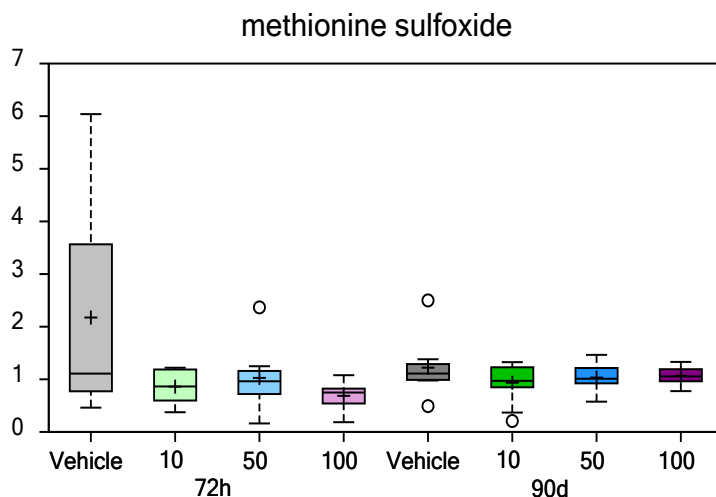
changes via licofelone treatment. I would like to include, in this report, some of the data that we find intriguing from this study:

1. *SCI-dependent increase in glutarate concentrations:* Glutaric acid, or glutarate, is a byproduct of amino acid metabolism, specifically of lysine and tryptophan. Pathologic production of glutarate can have cellular toxic effects. SCI causes an increase in glutarate concentration within the testis detectable by 72 hours post-injury. Interestingly, this increase can be detected in the testis of vehicle-treated subjects as late as 90 days post-injury. Licofelone treatment, particularly the 10 mg/kg concentration, appears to attenuate glutarate concentrations at 72 hours. This effect appears to be preserved as glutarate levels remain attenuates at 90 days in licofelone vs. vehicle-treated subjects.

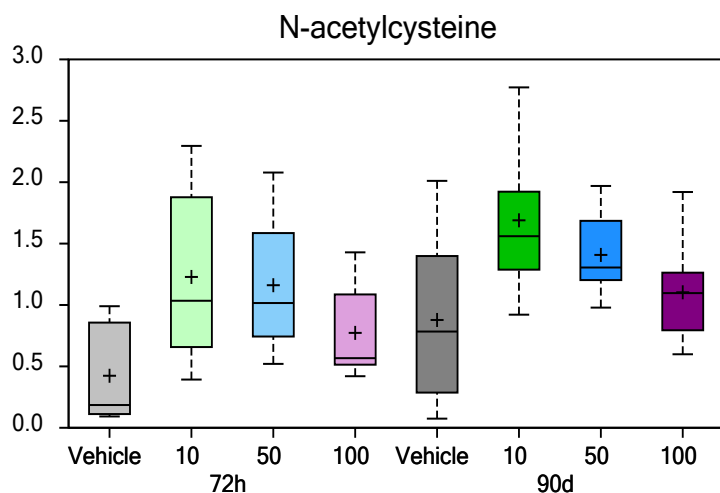


2. *Oxidative Stress:* Our prior studies have indicated the presence of elevated pro-oxidative conditions both within the testis (Aim 1) and spinal cord (not show here) during both acute and chronic time points. Our goal was to determine whether licofelone treatment could prevent such conditions within the testis following SCI. Here we describe some of the biochemical indices representing both pro- and anti-oxidative conditions as well as the effects of licofelone on these pathways.

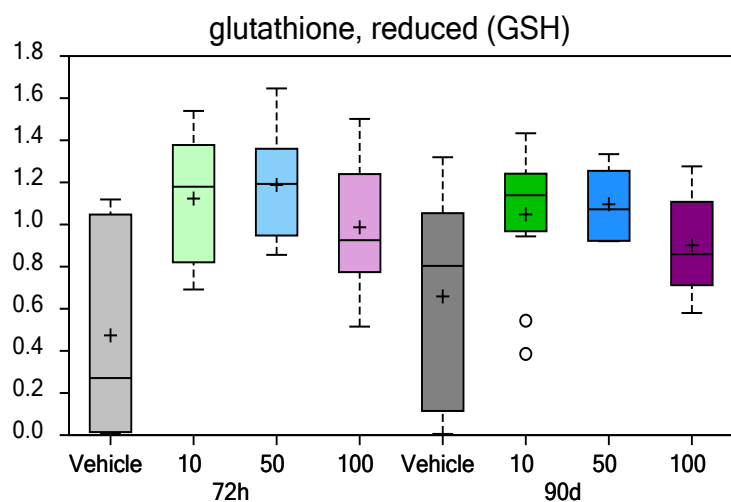
Methionine Sulfoxide: Oxidation of the sulfur motifs of methionine can indicate a pro-oxidative environment. We observe a dramatic increase in methionine sulfoxide in the testis by 72 hours post-SCI, suggestive of the establishment of a pro-oxidative environment. This observation is attenuated by licofelone at all three doses at this time point. By 90 days, methionine sulfoxide levels are reduced, suggestive that increased oxidation of methionine in the testis is a more acute event (see figure below).



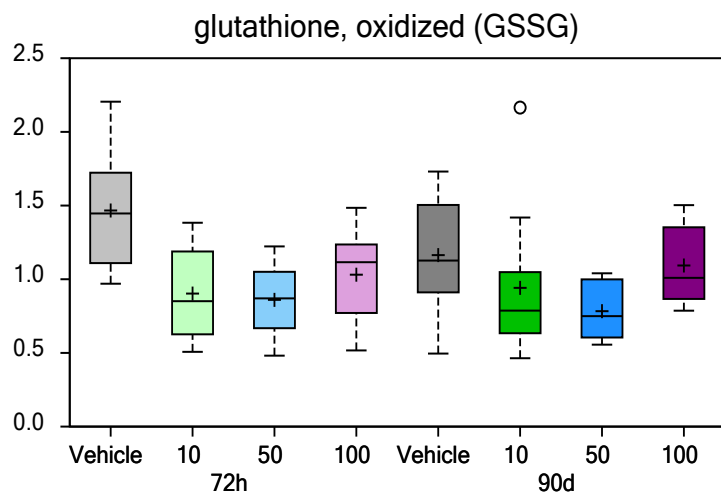
N-acetylcysteine (NAC): NAC is a cysteine metabolite with potent anti-oxidant properties. Acute licofelone treatment dramatically elevates NAC levels compared to vehicle-treated subjects by 72 hours. More intriguing, this effect is maintained following cessation of treatment when examined in subjects at 90 days post-injury.



Glutathione (reduced and oxidized): Glutathione is a component of a major anti-oxidant pathway in the body with elevated oxidized glutathione representing a pro-oxidative environment with reduced glutathione indicating greater anti-oxidant capacity. We observe a licofelone-dependent increase in levels of reduced glutathione at 72 hours post-SCI compared to vehicle treated. Of even greater interest, this effect is observed at 90 days post injury; long after cessation of licofelone treatment.



Similarly, we observe elevated concentrations of oxidized glutathione, suggestive of pro-oxidant conditions, at 72 hours post-injury whereas licofelone treatment results in decreased levels of oxidized glutathione; an effect observed out to 90 days post-injury (long after cessation of treatment).

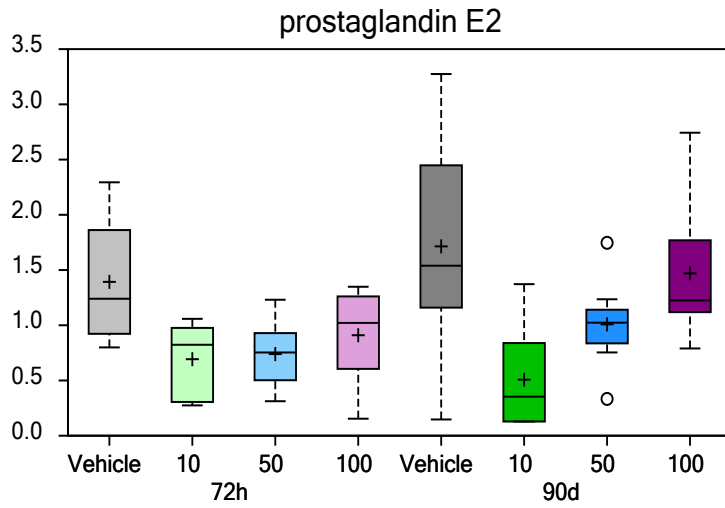


These metabolomic results focus almost exclusively on oxidative stress events; showing that SCI induces pro-oxidative conditions within the testes that can be attenuated via licofelone treatment. More importantly, with several of these mediators, the licofelone-mediated effect is observed long-after cessation of licofelone treatment; indicating a long-term, protective role of licofelone within the testes. We will continue to analyze the metabolomic data to identify other metabolic pathways pathologically altered by SCI and what effect licofelone may have as a beneficial therapeutic.

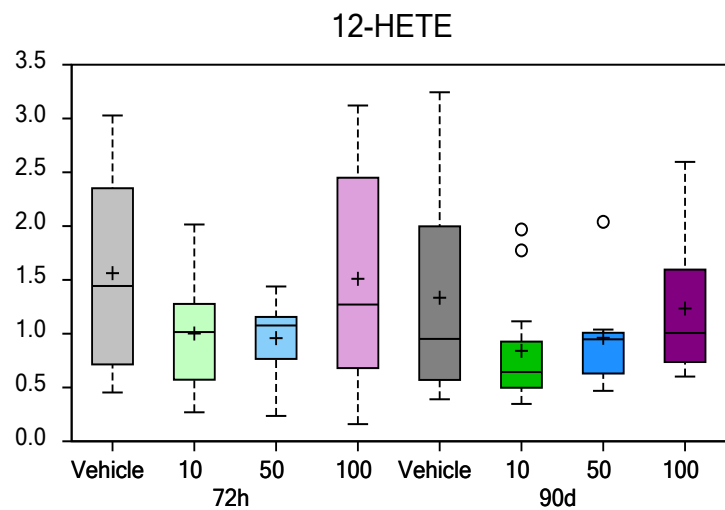
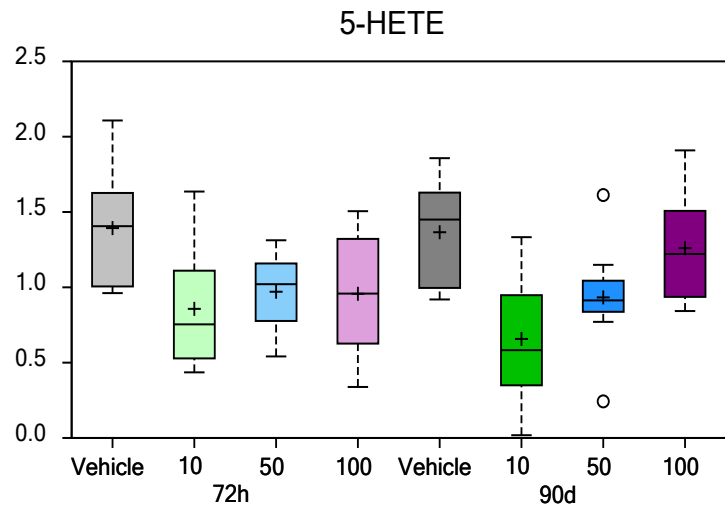
Aim 2 (Fourth Quarter): Progress in Aim 2 from the fourth quarter of year 2 continues to focus on our metabolomic analyses.

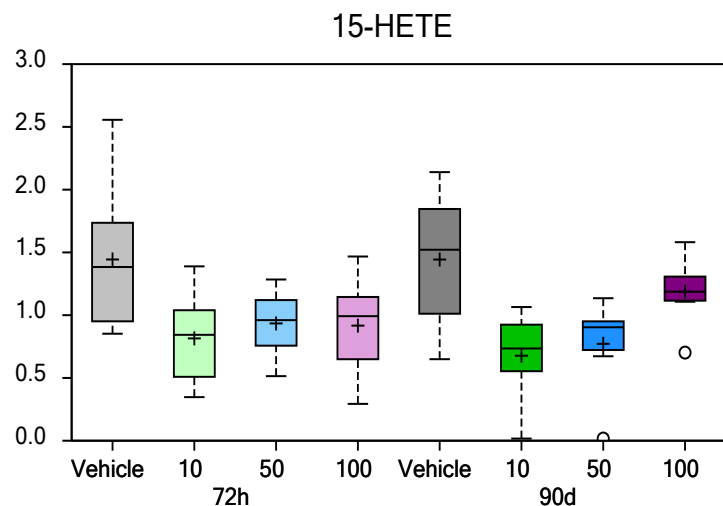
Arachidonic acid metabolism: A central hypothesis of our proposal was that SCI would elicit a profound inflammatory response within the testes of rats utilizing the arachidonic acid pathway. Our results, to this point, support this hypothesis (see below). More important, it appears that licofelone treatment produces an early but sustained attenuation of the production of pro-inflammatory, arachidonic acid-derived, metabolites. As part of our NCE request, we will be validating several of these mediators, particularly metabolites of cyclooxygenase and lipoxygenase production.

Prostaglandin E2 (PGE2): We observe an SCI-dependent increase in PGE2 by 72 hours post-SCI that, remarkably, is maintained in vehicle-treated subjects out to 90 days. Licofelone-treatment (once daily via oral gavage for 14 days starting 3 hours post-SCI) produced an attenuation of PGE2 production when analyzed at 72 hours post-injury. This effect was maintained at the 10 and 50 mg/kg licofelone treatment group when analyzed at 90 days, long after cessation of licofelone treatment.



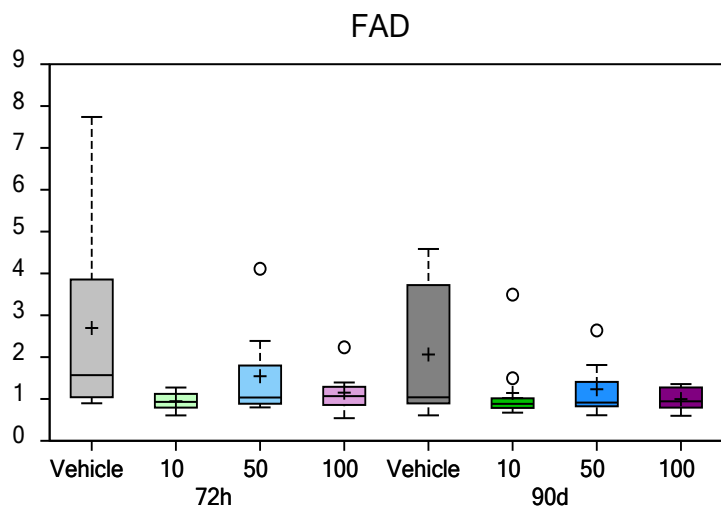
Other pro-inflammatory mediators such as 5-, 12-, and 15-HETE, created via lipoxygenase production, undergo elevated expression after injury but show a sustained reduction in expression following licofelone treatment.





Leukotriene B4 and the cysteinyl leukotrienes were not present at the time of this study on the Metabolome profile, however, we are currently attempting to assess the effects of injury and subsequent licofelone treatment on both subclasses of leukotriene mediators using commercially-available ELISA kits.

Flavin Adenine Dinucleotide (FAD): While FAD ratio (FAD vs. FADH₂) can be included in the list describing anti-oxidants, we choose to describe the metabolomic results here as FAD also plays crucial roles in energy production through the electron transport chain. We observe an increase in FAD concentrations at 72 hours that continues to be elevated out to 90 days. Licofelone treatment (at any of the utilized concentrations) lowers levels of oxidized FAD both during the acute, treatment period as well as during the chronic period following cessation of treatment. It is intriguing to speculate that such an effect may result in greater availability of FADH₂ which would be of use in suppressing oxidative stress as well as maintaining the integrity of the electron transport chain, and thus, assisting in the support of energy generation in the testes following SCI.



These results confirm that SCI induces a profound alteration in metabolic function within the testes that, in many situations, becomes a chronic condition. Furthermore, acute treatment with licofelone appears to reduce many pathological metabolic changes within the testes with these effects maintained long after cessation of treatment. During the No Cost Extension period, we are planning on confirming/validating many of these changes while also assessing the effects of injury/treatment on structural elements of the BTB. Finally, we are proposing to assess whether treatment with licofelone leads to preservation of sperm and sperm germ cells as this would suggest a licofelone-mediated preservation of male fertility; the overall goal of this entire proposal.

What opportunities for training and professional development has the project provided? As described in a previous progress report, Lt. Ryan Fortune, a member of the Texas National Guard and an MD/PhD student at UT-Health (and my laboratory) has been working on this project as part of his thesis work. His effort (but nothing else) had been covered by a UT-Health Training Grant. This project has given Lt. Fortune an excellent opportunity to explore a clinically-relevant, yet under explored problem that faces many men in both the civilian and military environment. Ryan approached Dr. Grill to work in Dr. Grill's laboratory to gain the PhD component of his MD/PhD program. Ryan has a strong interest in exploring neurotrauma as a specialty upon completion of his degree studies. As a member of the Grill laboratory, Ryan has attended class in clinical translation and has shadowed neurotrauma clinicians at Memorial/TIRR Hospital here in Houston. In addition, Ryan has had the opportunity to observe long-term patient care at TIRR Rehab Hospital, also here in Houston. Ryan has been trained by both Dr. Grill as well as Dr. Loose in execution of the rat spinal cord injury model as well as animal care and treatment. Ryan has also received extensive training in the analysis of data resulting from the metabolomic and genomic studies. Proposed validation studies will be performed by Ryan in order to complete his thesis work (tentatively by the end of July, 2015).

How were the results disseminated to communities of interest? A presentation describing the current status of the project was presented at the Annual Mission Connect on December 5, 2015 by Lt. Fortune. This symposium assembles investigators from the North Coast region of Texas who study spinal cord and traumatic brain injury.

What do you plan to do during the next reporting period to accomplish the goals? We propose to utilize banked tissue to validate multiple arachidonic acid-derived mediators, measures of oxidative stress, BTB structural proteins and indices of mature sperm marker presence to confirm both: 1) SCI-induced pathophysiology and 2) the protective effects of licofelone treatment in regards to attenuating metabolic and genomic changes and preserving structural integrity of the BTB as well as the survival of mature sperm. These studies will NOT require the addition of any further animals, but can be accomplished utilizing remaining, frozen, testes tissues.

Impact:

What was the impact on the development of the principal discipline of the project? Our studies, to date, confirm our original hypothesis that spinal cord injury results in pathological changes that negatively impact the blood testes barrier. We believe that the impact of our findings are nothing short of profound as, while it was previously known that SCI negatively impacted male fertility, potential reasons underlying such a pathological response have been relatively unexplored. We now know that SCI creates conditions that results in inflammation, oxidative stress and altered energy metabolism in the testes, and that, in many instances, these pathological conditions are maintained long after the initial trauma. Further, treatment with licofelone, a drug that has gone through phase III clinical trials for osteoarthritis in Canada and has been shown in our lab to effectively suppress inflammation in the damage spinal cord, attenuates many of the SCI-induced pathological events observed in the testes during the treatment period. Of greater significance, the attenuation of several SCI-dependent pathological changes to the metabolome were shown to be maintained long after cessation of the treatment; suggesting that acute intervention with licofelone may preserve testicular function over chronic periods. If we are able to demonstrate that this involves the promotion of enhanced sperm production/survival, this would represent a step in the enhancement of SCI patient's overall quality of life.

What was the impact on other disciplines?

This is a topic under review in the laboratory. We and others are/have reported that SCI causes a range of pathological, systemic changes. Based on our current results, we are encouraged to examine other organ systems/functions to determine whether licofelone treatment can provide long-term enhancement of function.

What was the impact on technology transfer?

Unknown at the moment, but under investigation with UT-Health's Office of Technology Transfer.

What was the impact on society beyond science and technology?

Nothing to report.

Changes/Problems: None during this performance period.

Changes/Problems (NCE period): For our proposed NCE application, we have requested funds to cover proposed validation studies that should be concluded by the end of September, 2015. It is worth noting here, however, that the PI of this project, Dr. Grill, has accepted a new faculty position at the University of Mississippi Medical Center and will begin there on February 1, 2015. Dr. Grill has requested that overall PI status of the project be shifted to current Co-PI, Dr. David Loose of UT-Health. Dr. Grill will stay on in a non-paid consultant role where he will continue providing direction and SCI-expertise to both Dr. Loose and Lt. Fortune to enable: 1) completion of this project and 2) completion of Lt. Fortune's thesis work. This request is currently pending with MEDCOMM.

Products:

Publications, conference papers, and presentations

Journal publications: None as of yet, however we are currently working on two manuscripts, one of which describes the initial pathophysiology and the second the effects of licofelone on pathological metabolic changes within the testes. We anticipate submitting the first manuscript by the end of March 2015 and the second by the end of June, 2015.

Presentations: A limited description of results to date was presented by Lt. Fortune at the Annual Mission Connect Symposium held here in Houston, Texas on December 5, 2014.

Technologies or techniques: None to report as of now.

Inventions, patent applications, and/or licenses: Nothing to report as of now, but we are in discussion with the UT-Health Office of Technology Transfer to discuss how to move licofelone forward as a novel treatment for SCI-dependent loss of male fertility.

Participants and other collaborating organizations:

What individual have worked on the project?

Ray Grill, Ph.D.

PI

Nearest person month worked: 3 months

Contribution to project: Design, assist with surgeries and tissue collection, data analysis

Funding Support: No change

David Loose, Ph.D.

Co-Investigator

0.6 months

Performed gene array studies and assists in data analyses from such studies

no change in funding support during this period

Lt. Ryan Fortune

Graduate Research Assistant

no effort during this period, though we are requesting 100% effort for Ryan during the NCE period.

Performed all spinal contusion injuries, dosing, assisted with tissue collection, preparation of tissues for transit to Metabolon, Inc. and to Dr. Loose' Genomics Core here at UT-Health. Has been working on this project as his thesis project.

Was covered on a UT-Health Training Grant until November 30, 2014.

What other organizations were involved as partners?

None.

Special reporting requirements:

Collaborative awards: N/A

Quad Charts: see attached

Appendices: I have attached a digital copy of Lt. Fortune's recent poster presentation at the Annual Mission Connect Consortium Symposium.



The blood-testis-barrier and male sexual dysfunction following spinal cord injury

W81XWH-12-1-0481/SC110183



PI: Raymond J. Grill

Org: UT-Health

Award Amount: \$257,812

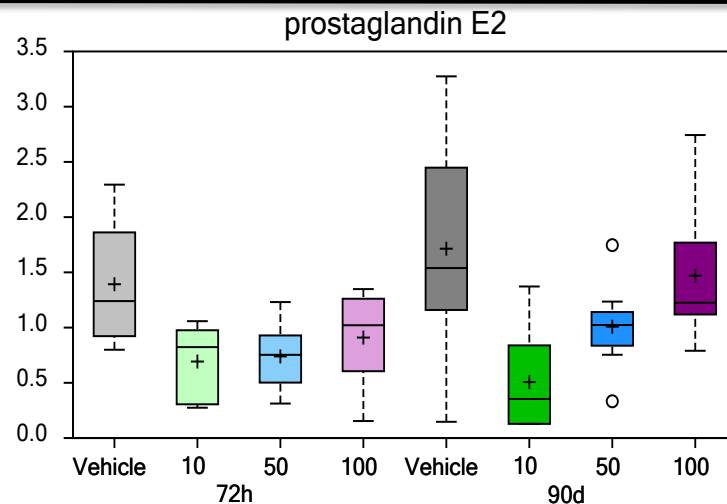
Study/Product Aim(s)

• **Specific Aim 1:** Explore the molecular, biochemical and structural changes that occur to the BTB as a function of time following the delivery of a clinically-relevant spinal contusion injury.

• **Specific Aim 2:** Determine whether treatment with the new generation anti-inflammatory drug, Licofelone, can protect BTB integrity and enhance germ cell and sperm viability over time following SCI.

Approach

Utilize a clinically-relevant rat spinal contusion model of injury to assess the early and long-term effects on the blood-testis-barrier as a mechanism underlying male infertility following spinal cord injury.



Accomplishment: SCI elicits pro-inflammatory prostaglandin production in the testes early and over a sustained period. Early licofelone treatment results in a sustained reduction in PGE2 production long after cessation of treatment.

Timeline and Cost

Activities	CY	12/13	13/14
This quarter: Started analysis of metabolomic profile in testes following SCI in animals treated with vehicle or licofelone			
Report licofelone mediated effects in countering pro-oxidant pathways			
Estimated Budget (\$K)		\$257,812	\$279,628

Goals/Milestones (Example)

CY12/13 Goal – Initiate Aim 1 of project

- ☒ Gain institutional approvals for all aspects of project
- ☐ Identify/hire individual to assist with project ☒
- ☐ train individual in surgical procedure, animal care ☒
- ☐ perform all animal surgeries outlined in Aim 1 ☒
- ☐ collect, store tissues; prepare samples and send out for metabolomic and gene array studies ☒

CY13/14 Goal – Complete Aim 2

- ☐ Analyze all data generated from Aim 1 to identify SCI-induced targets in testis tissues.
- ☐ Perform all surgeries/treatment studies ☒
- ☐ collect and process tissues ☒
- ☐ analyze tissues: ongoing!
- ☐ Determine validation targets to confirm metabolon results (NCE)

Budget Expenditure to Date

Projected Expenditure:

Actual Expenditure: \$

Updated: (12/22/2014)

Blood Testis Barrier Integrity in Spinal Cord Injury and the Protective Effect of Licofelone in a Sprague-Dawley Rat Model

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Spinal cord injury (SCI) results in devastating changes to almost all aspects of patient's lives. One symptom that remains largely uncharacterized is the development of male infertility. Erectile dysfunction and sensory loss can be significant, but are easily treated. SCI causes measurable pathology in the testis both acutely and chronically, leading to as of yet untreatable loss in sperm motility and viability. SCI has been shown previously to induce leukocytospermia, inflammatory cytokines, anti-sperm antibodies, and reactive oxygen species within the ejaculate. The Grill lab has shown that SCI causes a sustained disruption in the blood testis barrier, leading us to investigate further into the testes environment in SCI (Dulin et al, 2011). Using mRNA and metabolomics assessments, we have described the changes that occur within the testis over the course of injury. From 24 hours, 72 hours, 28 days, to 90 days post injury, the testis show an initial drop in normal sexual organ processes and initiation of an innate immune response that transitions to chronic low level immune activity. These data provide further evidence of both short- and long-term pathophysiology within the testes following SCI. In addition, these studies identify multiple, novel, avenues of exploration for treatment of male infertility in SCI.

Methods

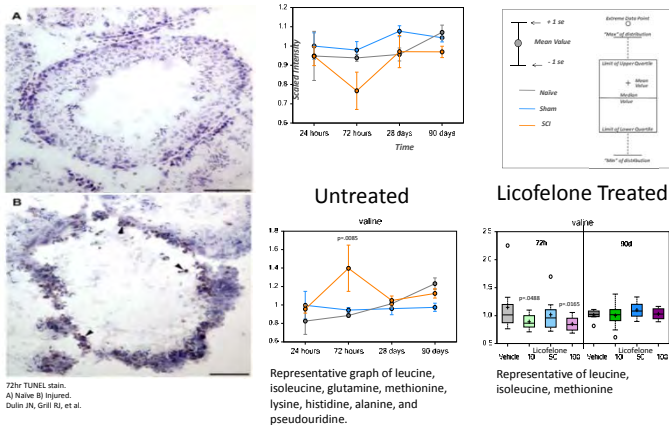


Adult, male, Sprague-Dawley rats received a spinal contusion injury at thoracic level 10 (T10) using 150 kdynes of force over a 1 second dwell time using the Infinite Horizons spinal impactor (Precision Systems and Instrumentation). Sham-injured subjects were anesthetized and received a spinal laminectomy at T10, but did not receive a spinal contusion injury. All injured subjects received appropriate post-operative care, including antibiotics, analgesic, hydration, and bladder care. To control for weaker injuries, subjects were examined using the non-invasive, Basso, Beattie, Bresnahan (BBB) open field locomotor test beginning on day 1 post-SCI. Briefly, this test scores rat hind limb function using a 21-point scale. Subjects that scored more than 2 on either day 1 or 2 of the study were excluded as such a score indicates an insufficient level of spinal damage.

Cohorts of eight injured, sham, and naive animals at the 24 hours, 72 hours, 28 days, and 90 days post-SCI were sacrificed and the testes immediately frozen for later analysis. Similar cohorts treated with licofelone were harvested at 72 hours and 90 days post-SCI. These treated cohorts were dosed by oral gavage once per day for 10 days starting 3 hours after injury with either 0 (vehicle), 10, 50, or 100 mg/kg of licofelone. To get a representative sample, testes were homogenized and separated into aliquots. We used a random sample from each animal's testis for RNA array analysis with Dr. David Loose and metabolomics assessment via Metabolon, who uses ultrahigh performance liquid chromatography and gas chromatography with mass spectrometry. Subsequent data was analyzed using ANOVA.

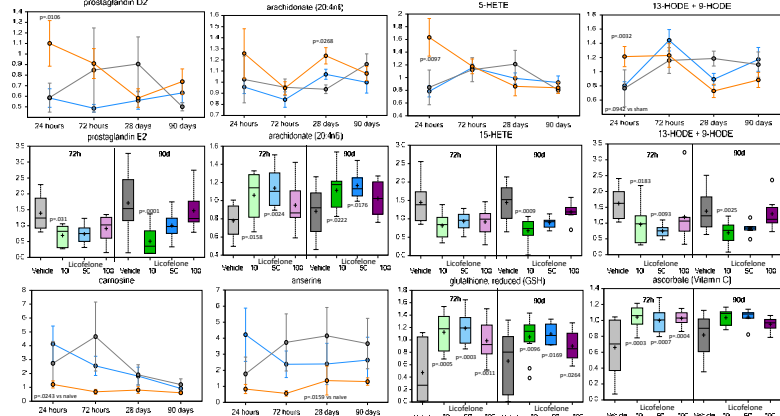
Cell Death in the Testes at 72 Hours

Our data shows significant increases in multiple amino acids, lysolipids, and nucleic acid degradation products at 72 hours. These data support the findings by Dulin et al, 2011, via TUNNEL staining that shows large amounts of cell death within the seminiferous tubules 72 hours after SCI. Licofelone treatment showed modest decreases in a few of these metabolites, suggesting that licofelone treatment has a protective effect after injury. If so, then that suggests that the inflammatory environment contributes to cell death in the testes after SCI.



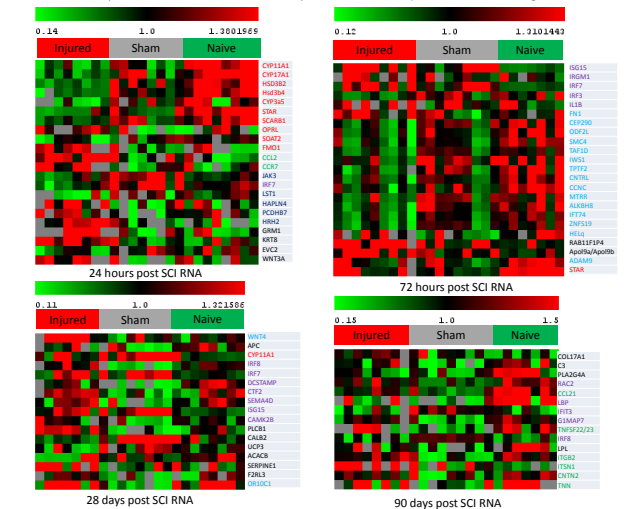
Metabolomic Analysis: Inflammation and Oxidative Stress in the Testes following SCI

SCI elicited pro-inflammatory and pro-oxidative events in the testes. However, many of these changes were trends and not statistically significant. Licofelone treatment elicits a suppression of these pathological events. Changes detected via gross metabolomic analysis will require individual, more sensitive, validation.



Heatmaps of Significant RNA Changes

RNA array data succinctly shows the progression of SCI's effects in the testes. At 24 hours we have a decrease in 10 **steroidogenesis** genes and the induction of **chemotactic** and **inflammatory** genes. At 72 hours we see a greater induction of **immune system** genes and a decrease in a massive amount of **cell cycle, DNA function, and sperm development** genes. In the chronic time points we continue to see elevated expression of **immune system** and **chemotactic** genes.



Potential Systemic Effects

Urea Cycle

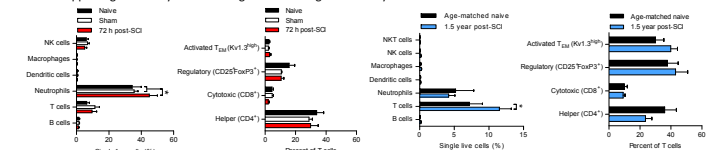
SCI caused an increase in urea and ornithine and a decrease in creatinine that recovers over time, all of which was somewhat attenuated by licofelone treatment. If localized to the testes, an alteration in the urea cycle at 72 hours could support SCI-dependent apoptosis and massive protein metabolism. However, the possibility of a systemic nitrogenous waste overload from either SCI induced mild liver toxicity or muscle tissue loss can not be ruled out.

Bile Acid Metabolism

Similarly, bile acid metabolism shows strong changes in the chronic setting that is strongly attenuated by an early 10 mg/kg dose of licofelone. This both gives us a more accurate effective dose range and provides stronger evidence to look into liver metabolism during SCI.

Cellular Response

Freshly harvested testes tissue was processed for immunophenotyping via fluorescence-activated cell sorting (FACS) for both acute (72 hour) and chronic (1.5 years) injured animals. Acutely, we see an infiltration of T lymphocytes, further indicating an acute inflammatory environment. Chronically, we see a continued immune response in the form of T cells, suggestive of an autoimmune response and further supporting the theory of SCI causing an immunological infertility.



It has been suggested that spinal trauma elicits both temporal and spatial changes occurring over a prolonged time frame. These data suggest that such SCI-induced changes are not restricted to the injured spinal cord, but can occur systemically; in this instance, within the testes. We demonstrate that early treatment with licofelone can attenuate many of these pathological changes. Since most of the inflammatory changes occurred at 24 hours, future experiments looking at licofelone's effect on the inflammatory environment will focus there, although oxidative stress continues chronically. It would be of considerable clinical interest/relevance to determine whether delayed licofelone treatment could reverse chronic pro-oxidative pathologies in the testes of chronically-injured subjects and return lost sexual function. Further evaluating this attenuation through histological assessment, analyzing RNA changes within the testes of treated animals, and demonstrating any functional improvement in fertility parameters are all planned subsequent next steps.

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Dulin JN, Moore ML, Gates KW, Queen JH, Grill RJ. Spinal cord injury causes sustained disruption of the blood-testis barrier in the rat. *PLoS one*. 2011;6(1):e16456. PubMed PMID: 21129806.